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Gene expression profiling of the short-term adaptive response to acute caloric restriction in liver and adipose tissues of pigs differing in feed efficiency

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Abstract

Residual feed intake (RFI) is a measure of feed efficiency, in which low RFI denotes improved feed efficiency. Caloric restriction (CR) is associated with feed efficiency in livestock species and to human health benefits, such as longevity and cancer prevention. We have developed pig lines that differ in RFI, and we are interested in identifying the genes and pathways that underlie feed efficiency. Prepubertal Yorkshire gilts with low RFI (n = 10) or high RFI (n = 10) were fed ad libitum or fed at restricted intake of 80% of maintenance energy requirements for 8 days. We measured serum metabolites and hormones and generated transcriptional profiles of liver and subcutaneous adipose tissue on these animals. Overall, 6,114 genes in fat and 305 genes in liver were differentially expressed (DE) in response to CR, and 311 genes in fat and 147 genes in liver were DE due to RFI differences. Pathway analyses of CR-induced DE genes indicated a dramatic switch to a conservation mode of energy usage by down-regulating lipogenesis and steroidogenesis in both liver and fat. Interestingly, CR altered expression of genes in immune and cell cycle/apoptotic pathways in fat, which may explain part of the CR-driven lifespan enhancement. In silico analysis of transcription factors revealed ESR1 as a putative regulator of the adaptive response to CR, as several targets of ESR1 in our DE fat genes were annotated as cell cycle/apoptosis genes. The lipid metabolic pathway was overrepresented by down-regulated genes due to both CR and low RFI. We propose a common energy conservation mechanism, which may be controlled by PPARA, PPARG, and/or CREB in both CR and feed-efficient pigs.

Disciplines

Agriculture | Animal Sciences | Bioinformatics | Biometry | Computational Biology | Computational Neuroscience

Comments

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Gene expression profiling of the short-term adaptive response to acute caloric restriction in liver and adipose tissues of pigs differing in feed efficiency

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microarray; transcriptional profiling; ESR1; residual feed intake; peroxisome proliferator-activated receptor a; peroxisome proliferator-activated receptor g; cAMP response element binding; protein

GENETIC MECHANISMS THAT CONTROL feed intake (FI) and feed efficiency are not well understood. Differences in feed efficiency arise due to factors such as variations in body composition, feeding patterns, digestibility, activity, thermoregulation, and tissue metabolic rates (68). Residual feed intake (RFI) has been broadly accepted as a reliable method of measuring feed efficiency and is defined as the feed consumed above or below what is required for growth and maintenance (47, 54). Pigs with low RFI (LRFI) consume less food than the population average without a significant loss in growth parameters such as body weight and composition, and therefore, they are more feed efficient. Our group has successfully developed pig lines that differ in RFI up to 124 g/day without significant change in the body composition, with an estimated heritability for RFI of 0.33 (12). The physiology underlying RFI differences has been studied mainly in poultry and in beef cattle, in which whole-genome SNP analyses and microarray approaches have been undertaken (6, 9, 74). For example, transcriptomic analysis of liver biopsies from Angus bulls identified 163 differentially expressed genes between animals with high and low RFI (16). These genes represented several cellular pathways, such as growth, proliferation, protein synthesis, lipid metabolism, and carbohydrate metabolism (16).

Efficient feed utilization has also been achieved with caloric restriction (CR) in cattle and chickens (67, 69). The motivation to understand biological mechanisms underlying response to CR extends beyond feed efficiency in livestock species. Caloric restriction prolongs lifespan in virtually all species, including mammals, and recent reports suggest that CR is the most compelling cancer-prevention regimen in the carcinogenesis models (2, 38). Translation of the CR phenomenon to human health is critical, considering that obesity, a major risk factor for several types of cancers and age-associated chronic diseases, is alarmingly increasing in the Western world (80).

Transcriptional profiling of CR to elucidate pathways involved in longevity-promoting mechanisms in rodents has been investigated (76, 83, 85); however, the pig is a better suited model for the human energy homeostatic system than rodents and has contributed to improved knowledge of human metabolic disorders, such as obesity and diabetes (59, 72). Understanding efficient feed utilization in pigs will also lead to improved agricultural economy, as pork is used as a major human food source worldwide, and the cost of feed amounts to the largest variable cost in pork production, making up 68% of the total variable cost (33). Significant advances have been made in swine transcriptomics in recent years (79), and gene expression profiling has been employed in several porcine tissues, including adipose and liver tissues (52).

Several transcription factors (TF) and nuclear receptors are known to mediate the response to CR (22), and they regulate specific metabolic adaptations within each peripheral organ and coordinate intertissue communication for energy homeostasis. Previous studies have established regulatory roles for several TF in fat and/or liver tissues, such as CCAAT/enhancer binding protein alpha (15), hypoxia inducible factor 1, alpha subunit (HIF1A) (42), peroxisome proliferator-acti-

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vated receptors gamma (PPARG) (82), and alpha (PPARA) (57). However, previous large-scale transcriptional profiling reports on CR or RFI (16, 73, 83) have not included global analysis of key regulatory TF that may be responsible for the differentially expressed (DE) genes.

Our objective was to identify pathways and transcriptional networks of genes responding to CR or to differences in RFI using transcriptional profiling of over 24,000 genes in key metabolic tissues of pigs that is corroborated by blood metabolite analyses. Our hypothesis was that such results may implicate candidate genes to improve feed efficiency in pigs, as well as elucidate some of the longevity promoting mechanisms associated with CR. We report 6,114 genes in adipose tissue and 305 genes in liver to be DE ($q \leq 0.05$) due to 8-day CR and 311 fat genes and 147 liver genes to be DE due to differences in RFI. Major potential regulators of genes responding to CR were identified by assessing the known regulatory connections of TF to the DE genes. This study reports *1)* the first global transcription profiling study on the effects of CR and differences between high and low RFI groups of pigs, *2)* identification of key TF in the global response to CR or to RFI group differences, and *3)* analysis of shared genes and biological pathways between the responses to CR and RFI group differences.

MATERIALS AND METHODS

Animals and Feeding Treatments

Six-month-old Yorkshire gilts from either a line selected for three generations for improved feed efficiency, based on low RFI (12) or from a control line were used. Animals were fed ad libitum (AL) standard swine diet with 20% crude protein until weaning (Kent Feeds, Muscatine, IA) and with 16–17.5% crude protein diet after weaning (Mid-State Milling, State Center, IA). To exclude potential effects of genotype for MC4R D298N, which has been reported to be associated with FI, backfat, and growth (45), pigs were genotyped for this MC4R variant and 90 heterozygous gilts, 45 from each line, were placed on the FIRE electronic feeders starting at ~35 kg until pigs weighed ~75 kg. Subsequently, pigs were evaluated for average daily FI, average daily gain (ADG), and RFI using procedures described previously (12). Ten animals with the highest RFI from the control line and 10 animals with the lowest RFI from the selection line were randomly assigned to feeding treatments and individually penned. Henceforth, we refer to the RFI differences between high and low RFI groups as the RFI difference. Starting at 0600 on *day 1*, gilts were fed AL with continuous access to feed until ~0800 on *day 9* or were fed twice daily with feed equivalent to 80% of maintenance energy requirements for 8 days in a randomized complete block design with a 2 (RFI group) × 2 (feed) factorial arrangement of treatments. Maintenance energy requirements were estimated as 110 kcal of digestible energy per kg of metabolic weight, which was defined as body weight raised to the 0.75 power (62a). Digestible energy content of the feed was 3,600 kcal/kg. Blocks were defined as animals that underwent treatment on the same day, and each block contained at least one animal from each combination of RFI group and feed treatment (see Supplemental Table 1 in the online version of this article). A total of five animals were used for each combination of RFI group and feed treatment. Tissues from 10 pigs were harvested in the fall and from another 10 in the following spring. All animals received water AL. On *day 9* (0900–1130), pigs were killed by electric stunning and exsanguinations. Samples of hepatic tissue and the 10th rib middle layer of backfat were rapidly collected, frozen in liquid nitrogen, and stored at −80°C until RNA isolation. Blood samples were collected during postmortem exsanguinations. Blood samples were allowed to clot at 4°C overnight, centrifuged at 1,200 *g* for 30 min, and serum was collected and stored at −20°C. The protocol for the animal experiments was reviewed and approved by the Institutional Animal Care and Use Committee of Iowa State University (12-04-5797-S).

Feed Intake, Body Weight, Backfat, Loin Eye Area, and Blood Parameters

During the 8-day treatment period, the FI of each pig was recorded daily. Pretreatment and posttreatment body weight, 10th rib backfat depth, and loin eye area were measured on *days 0* and *8* around 1600–1800 (*day 1* was designated as the start of feeding treatment). Backfat depth was measured by ultrasound (12) using an Aloka 500V SSD ultrasound instrument fitted with a 3.5-MHz, 12.5-cm, linear-array transducer (Corometrics Medical Systems, Wallingford, CT). Posttreatment serum samples were assayed for concentrations of glucose (Hexokinase assay, Roche Diagnostics, Indianapolis, IN, USA), insulin (Porcine Insulin Kit, cat. no. PI-12K; Linco Research, St. Charles, MO), triglyceride (TG; cat. no. 236–99, Diagnostics Chemicals, Oxford, CT), nonesterified fatty acids (NEFA, HR Series-NEFA HR-2; Wako Diagnostics, Richmond, VA), cortisol (Cortisol I^{125} RIA Kit, cat. no. 07-221102, MP Biomedicals), leptin (Multispecies Leptin Kit, cat. no. XL85-K, Linco Research), thyroxine, and triiodothyronine (T4, cat. no. 06B-254030 and T3 cat. no. 06B-253216, MP Biomedicals, Irvine, CA).

RNA Isolation and Microarray Hybridization

Total RNA was isolated and purified from liver and adipose tissues using the Qiagen RNeasy midi kit and the Qiagen RNeasy lipid tissue kit (Qiagen, Valencia, CA). Quality and quantity of RNA were determined by using the Agilent 2100 Bioanalyzer (Foster City, CA). Average RNA integrity numbers were 9.5 ± 0.3 (means ± SD) for liver RNA and 8.4 ± 0.7 for fat RNA samples. Microarray target sample processing, target hybridization, washing, staining, and scanning steps were completed according to manufacturer's instructions (Affymetrix, Santa Clara, CA) at two different times (fall and spring). RNA from liver and adipose tissue samples of 10 pigs that were harvested in the fall were hybridized in the fall, with exception of RNA samples from two pigs that were hybridized in the spring along with the RNA from 10 pigs that were harvested in the spring. Briefly, 10 μg of total RNA from liver or adipose tissue was used to synthesize cDNA using a one-cycle cDNA synthesis kit. Resulting cDNA from each sample was used to transcribe biotinylated cRNA by T7 RNA polymerase and further fragmented and applied to the Affymetrix GeneChip Porcine Genome Array (hereafter called the Porcine GeneChip) that contains 24,123 probe sets. Following hybridization at 45°C for 16 h, the array was washed and stained with streptavidin-phycoerythrin at an Affymetrix GeneChip Fluidics Station 450 and fluorescent signals were scanned using an Affymetrix GeneChip Scanner 3000 (1). Each tissue was assigned to one fluidics station to remove potential station effects for within-tissue comparisons, and four modules within each station were intentionally confounded with the blocks.

Transcriptome

The transcriptome for each tissue was established as described previously (52). The probe-pair data were used to determine the detection call by the modified Wilcoxon signed rank test of the MAS 5.0 software (51). For each tissue, probe sets with absent calls for all replicates in all treatments were removed, and the remaining probe sets were declared as expressed and established the transcriptome for that tissue.

Statistical Analyses

Animal performance and blood parameters. Posttreatment body weight, backfat depth, loin eye area, pretreatment RFI, ADG, FI

during treatment, and blood parameters were analyzed by a mixed linear model with RFI group, feed treatment, and their interactions as fixed effects, block as a random effect, and their pretreatment values (except for RFI) as a linear covariate. Analyses were performed by the mixed procedure of SAS/STAT software ver. 9.1.3 (SAS Institute, Cary, NC).

Gene expression. CEL files generated by the Affymetrix GeneChip Operating Software at the GeneChip Facility at Iowa State University were used as the raw data for statistical analysis. Data were deposited in the NCBI Gene Expression Omnibus (series accession no. GSE18359; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18359), and data quality was examined with BioC version 2.0 (31) in R (66). All 24,123 probes sets, including those with absent calls, were analyzed. Probe level measures were summarized into probe set level expression measures using the "simpleaffy" package (84) in R, according to the MAS 5.0 algorithm (1). However, the final step of aligning the trimmed means on the log scale was replaced by median centering normalization (86), i.e., subtracting the median expression from all expression measures on the logarithmic scale for each chip separately. Because the mean within-chip variability was different between the two sets of chips that were hybridized in two time periods, scale normalization (86) was further applied to match the within-chip median absolute deviations (MADs) to the geometric mean of MADs across all chips by a multiplicative scaling on the logarithmic scale for each chip. Both the median centering and the scale normalization were performed for each tissue separately.

To determine the proper model for analysis of the expression data, 405 candidate mixed linear models were fitted to the normalized expression measures for each tissue and each gene separately using the mixed procedure in SAS/STAT software ver. 9.1.3 (SAS Institute Cary, NC). All candidate models included RFI group, CR, and their interaction as fixed effects, but differed from each other by including any combination of the following nuisance factors in the model: *1)* tissue harvest season (fall or spring), with or without interaction with other treatment effects of interest, was included as fixed or random effect, or excluded from the model; *2)* blocks of pigs in each harvesting season (four levels) were broken up into five levels by coding as an additional group consisting of two pigs that were harvested in fall but hybridized at the same time as the pigs that were harvested in spring; and the resulting five-level factor was included as fixed or random effect, or excluded from the model; *3)* chip hybridization day (five levels), *4)* the process batch during washing of the chips (two levels) on the day after hybridization, and *5)* module (four levels) in the Fluidics Station 450 were each included as fixed or random effects, or excluded from the model. On the basis of overall evaluation of gene-averaged SAS reported information criteria, the gene-averaged standardized prediction sums of squares from leave-one-out cross-validation, experimental design, and histograms of *P* values of contrasts of interest, the selected model included harvesting season, chip hybridization day, and block of pigs (five levels) as random effects for both tissues. For fat, since the *P*-value histogram indicated a nondetectable interaction between RFI group and CR, this interaction term was dropped from the model.

In the selected model for each tissue, variance components were estimated with the method of residual maximum likelihood under nonnegativity boundary constraints. Fixed effect estimates and least squares means were then obtained using generalized least squares, and fixed effects contrasts were tested using Wald-type *F*-tests with Kenward-Roger's correction (44). The positive false discovery control procedure (77) was used on each set of *P*-values of contrasts of interest to estimate *q* values (i.e., positive false discovery rate). Genes with *q* values ≤ 0.05 were declared to be DE as a result of caloric restriction and *q* values ≤ 0.2 were declared to be DE as a result of RFI. A less stringent threshold was used for RFI because of the lower power of this aspect of the experiment.

Affymetrix probe annotation. To obtain orthology information and improved annotation of the Porcine Genechip probesets, Affymetrix consensus sequences were used with BLASTN against the well-curated NCBI RefSeq database. Highest scores were used with a conservative cutoff of 1e-10 for the E-value. In total, 17,798 (73.8% of all) probesets were assigned RefSeq annotation (18).

Pathway analysis with gene ontology and Kyoto Encyclopedia Genes and Genomes. Genes in liver and subcutaneous fat that were DE because the CR ($q \leq 0.05$) or RFI group ($q \leq 0.2$) were divided into genes that were downregulated or upregulated as a result of caloric restriction, creating eight categories of genes. Using DAVID, an open-access Web-based functional annotation and clustering program (21), the eight categories of genes were analyzed for overrepresented ($P \leq 0.05$) Biological Process categories based on Gene Ontology (GO) or Kyoto Encyclopedia Genes and Genomes (KEGG) pathway categories (63). The *P* values for overrepresentation were computed by a modified Fisher's exact test, using the transcriptome for each tissue as background. The biological process categories were clustered using Functional Annotation Clustering (21), where the enrichment score for each cluster was computed as the negative log of the geometric mean of *P*-values in the cluster.

Pathway studio analyses. Four categories of gene lists made of DE genes in fat or liver tissues due to CR ($q \leq 0.05$) or RFI ($q \leq 0.2$) were analyzed with Pathway Studio 5.0 (63), a text-mining tool that detects relationships among genes, proteins, cell processes, and diseases as recorded in the PubMed database (Ariadne Genomics, Rockville, MD). Common regulatory transcription factors and nuclear receptors of DE genes were obtained based on reported (PubMed) interactions, which were defined as regulation, direct regulation, binding, and/or promoter binding. For each regulatory TF that satisfied these criteria, target DE genes were counted. Because of the very large size of the list of genes DE as a result of CR in fat, we tested in Pathway Studio only those genes with a fold change equal or greater than two.

Real-time quantitative PCR for verification of DE genes. Real-time quantitative PCR (qPCR) was used to verify differential expression due to either CR or RFI for eight genes in each tissue. Total RNA was isolated from backfat of pigs that were assayed using the Porcine Genechip as described above and reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) (20). Real-time PCR was performed in duplicate using 100 ng cDNA (RNA equivalent) per 25-μl reaction or per well with the Brilliant kit (Stratagene, La Jolla, CA) on Bio-Rad MyiQ Single Color real-time PCR Detection System (Bio-Rad, Hercules, CA). All probes and primers for real-time TaqMan PCR were designed (see Supplemental Table 2 in the online version of this article) using Primer Express 2.0 (Applied Biosystems, Foster City, CA), as previously described (20). The probes contained 3′ Iowa Black FQ quencher and 5′ 6-FAM reporter (Integrated DNA Technologies, Coralville, IA). The qPCR conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min, then 4°C. Because variation in the expression of commonly used housekeeping genes such as *GAPDH*, *SDHA*, *RPL32*, and *YWHAZ* (81) were observed in either a tissue or treatments in the Porcine Genechip, we normalized gene expression based upon the constant amount of RNA and cDNA amplified. This method has been proposed to be the most reliable standardization of quantitative measurement of mRNA expression, provided that accurate estimation of total RNA is made with tools such as Agilent Bioanalyzer (11).

Quantification of gene expression was analyzed as previously reported (20). Briefly, C_t values averaged across duplicate readings were analyzed by a mixed linear model with feed treatment, RFI, and their interactions as fixed effects, and block as a random effect, using the Mixed procedure of SAS/STAT software ver. 9.1.3 (SAS Institute, Cary, NC). A value of $P \leq 0.05$ was considered statistically significant for CR or RFI group effect. Fold change in expression was calculated as $2^{\Delta C_t}$ for each gene, where ΔC_t is the difference between least square mean C_t values for the CR and AL groups.

Table 1. *Body weight, average daily gain, feed intake, RFI, backfat depth, and loin eye area in pigs fed ad libitum or CR and that have high and low RFI*

	HRFI		LRFI				
	Ad Libitum Feed	Caloric Restriction	Ad Libitum Feed	Caloric Restriction	*P* Value RFI	*P* Value CR	*P* Value RFI × CR
Body weight, kg							
Pretreatment	84.21^{a}±2.99	82.92^{a}±2.85	79.66^{a}±2.99	78.85^{a}±2.99	6.9E-02	6.4E-01	9.2E-01
Posttreatment	88.72^{a}±0.61	76.44^{b}±0.55	89.17^{a}±0.61	77.20^{b}±0.60	3.5E-01	4.9E-13	7.9E-01
Average daily gain, kg/day							
Pretreatment	0.66^{b}±0.03	0.75^{a}±0.03	0.70^{a,b}±0.03	0.75^{a}±0.03	4.4E-01	9.2E-03	3.9E-01
During treatment	0.92^{a}±0.10	−0.62^{b}±0.08	1.04^{a}±0.09	−0.50^{b}±0.09	1.5E-01	1.3E-10	9.9E-01
Pretreatment RFI, kg/day	0.15^{a}±0.04	0.15^{a}±0.04	−0.14^{b}±0.04	−0.15^{b}±0.04	2.8E-06	9.2E-01	8.5E-01
Feed intake (kg/d)							
Pretreatment	1.68^{a}±0.07	1.80^{a}±0.06	1.40^{b}±0.07	1.44^{b}±0.07	3.0E-05	1.5E-01	5.2E-01
During treatment	2.83^{a}±0.06	0.61^{b}±0.07	2.87^{a}±0.07	0.80^{b}±0.07	2.3E-01	1.3E-16	2.1E-01
Backfat depth, mm							
Pretreatment	15.46^{a}±0.99	15.21^{a}±0.91	12.07^{b}±0.99	12.84^{a,b}±0.99	8.8E-03	7.9E-01	6.1E-01
Posttreatment	17.03^{a}±0.55	12.48^{c}±0.51	14.69^{b}±0.56	11.51^{c}±0.55	1.5E-02	1.5E-06	1.9E-01
Loin eye area, mm^2							
Pretreatment	3324^{a}±179.1	3234^{a}±165.2	3011^{a}±179.1	3349^{a}±179.1	5.6E-01	4.6E-01	2.3E-01
Posttreatment	3399^{a,b}±107.3	3138^{b}±99.6	3541^{a}±113.5	3302^{a,b}±107.9	1.2E-01	1.7E-02	9.1E-01

Values are least square means ± SE pooled across treatments; $n = 5$ per residual feed intake (RFI) by feeding treatment combination. HRFI, high residual feed intake; LRFI, low residual feed intake. Least square means within the same line that do not share the same superscript letter are significantly different at $P < 0.05$.

RESULTS

Caloric Restriction and RFI Effects on Body Weight, ADG, FI, Backfat, Loin Eye Area, and Serum Metabolites

The effects of RFI group and 8-day CR on pretreatment RFI and pretreatment and posttreatment body weight, ADG, FI, backfat, loin eye area are summarized in Table 1. The average RFI of the LRFI group was 0.3 kg/day less than that of the high RFI group (HRFI, $P < 0.0001$). The LRFI pigs tended to weigh less than the HRFI pigs at the beginning of treatment (LRFI = 79.2 kg, HRFI = 83.6 kg, $P = 0.07$). Pretreatment backfat depth was 2.9 mm less in the LRFI group than the HRFI group ($P = 0.009$), without a significant pretreatment loin eye area difference ($P = 0.56$). At the end of the 8-day feeding treatment, the body weights did not significantly differ between LRFI and HRFI pigs ($P = 0.35$); however, the backfat depth was less in the LRFI than the HRFI pigs ($P = 0.015$).

Eighty percent of maintenance caloric requirement was roughly equivalent to 25% of the AL feed, which was ~2.85 kg/day. Pigs weighed ~81.4 kg before the feeding treatment, and their body weight ($P = 0.64$), backfat depth ($P = 0.8$), and loin eye area ($P = 0.46$) did not significantly differ between CR and AL groups. During the treatment, AL pigs gained 0.98 kg/day, whereas CR pigs lost 0.56 kg/day (AL vs. CR, $P < 0.0001$), which resulted in the CR pigs weighing ~12 kg less than the AL fed pigs ($P < 0.0001$) at the end of treatment. The CR group's posttreatment backfat was 3.9 mm ($P < 0.0001$) and loin eye area was 250 mm^2 ($P = 0.017$) less than those of AL group.

Several blood parameters were measured in the posttreatment serum samples, and these results are shown in Table 2. Serum concentration of NEFA tended to be higher in the CR than the AL group ($P = 0.075$). Thyroxine concentration was 22.6% lower in the CR than the AL pigs ($P = 0.023$), and the triiodothyronine concentration tended to be 18.5% lower in the CR than the AL pigs ($P = 0.076$). No significant effect of CR was noted for serum concentrations of glucose, insulin, TG, cortisol, and leptin. The serum triiodothyronine concentration in LRFI pigs was 31% higher than that of HRFI pigs ($P = 0.024$). The effect of RFI was not significant for serum concentrations of glucose, insulin, TG, thyroxine, cortisol, and leptin. A significant interaction between RFI and CR was observed for the serum concentration of leptin ($P = 0.027$), in which leptin concentration tended to decrease in response to CR in the HRFI group, but the opposite trend was present in the LRFI pigs.

Table 2. *Blood parameters in pigs fed ad libitum or CR and that have high or low RFI*

	HRFI		LRFI				
	Ad Libitum Feed	Caloric Restriction	Ad Libitum Feed	Caloric Restriction	RFI *P* Value	CR *P* Value	RFI × CR *P* Value
Glucose, mg/dl	85.9^{a}±7.1	92.4^{a}±6.7	99.1^{a}±7.1	98.7^{a}±7.1	1.1E-01	6.0E-01	5.8E-01
Insulin, ng/ml	0.21^{a}±0.05	0.19^{a}±0.05	0.11^{a}±0.05	0.19^{a}±0.05	3.3E-01	6.0E-01	3.8E-01
NEFA, mEq/l	0.19^{a}±0.04	0.29^{a}±0.03	0.22^{a}±0.04	0.26^{a}±0.04	9.7E-01	7.5E-02	4.1E-01
TG, mg/dl	28.0^{a}±6.0	32.2^{a}±5.5	28.3^{a}±6.0	23.9^{a}±6.0	4.9E-01	9.8E-01	4.9E-01
T4, μg/dl	5.11^{a}±0.47	4.28^{a,b}±0.43	5.09^{a}±0.47	3.61^{b}±0.47	4.7E-01	2.3E-02	4.9E-01
T3, ng/dl	103.5^{a,b}±12.80	77.9^{b}±11.92	127.4^{a}±12.80	110.2^{a,b}±12.80	2.4E-02	7.6E-02	7.2E-01
Cortisol, ng/ml	19.78^{a}±12.5	22.31^{a}±11.8	13.55^{a}±12.5	22.09^{a}±12.5	7.5E-01	5.8E-01	7.7E-01
Leptin, ng/ml	4.37^{a}±0.76	3.31^{a}±0.43	2.84^{a}±0.45	4.05^{a}±0.63	5.4E-01	9.2E-01	2.7E-02

Values are least square means ± SE pooled across treatments, $n = 5$ per RFI by feeding treatment combination. NEFA, nonesterified fatty acids; TG, triglycerides; CR, caloric restriction. Least square means within the same line that do not share the same superscript letter are significantly different at $P < 0.05$.

Caloric Restriction and RFI Effects on Expression of Genes in Fat and Liver

Of 24,123 probe sets evaluated by microarray analysis, 20,058 and 18,787 provided data indicating that the transcripts represented by these probe sets were expressed in fat and liver tissue, respectively. In response to an 8-day CR treatment, 6,114 transcripts in fat were DE ($q \leq 0.05$, $P \leq 0.024$), of which 2,845 were upregulated and 3,269 were downregulated (see Supplemental Table 3*A* in the online version of this article). In liver, 305 transcripts were identified to be DE ($q \leq 0.05$, $P \leq 0.0009$), of which 156 were upregulated and 149 downregulated (Supplemental Table 3*B*). We considered transcripts with false discovery rate less or equal to 20% ($q \leq 0.2$) to be DE as a result of difference between RFI groups. Because of RFI difference between groups, 311 transcripts in fat ($P < 0.003$, Supplemental Table 3*C*) and 147 transcripts in liver ($P < 0.0015$, Supplemental Table 3*D*) were declared to be DE ($q \leq 0.2$). An improved annotation of probesets on the Affymetrix GeneChip Porcine Genome Array (18) assigned gene names (BLASTN expectation score < 1E-10) to 84% and 81% of all DE transcripts due to either CR or RFI in liver and in fat, respectively. For simplicity, the changes in RNA levels detected by these annotated probe sets are referred to as gene expression differences for the remainder of the article.

Microarray Data Validation by qPCR

Expression patterns were verified by qPCR for seven genes in either fat or liver, which were predicted to be DE due to CR ($q \leq 0.05$); these were selected to represent the lipid biosynthetic pathways in subcutaneous adipose tissue and gluconeogenesis in the liver (Fig. 1A). Similarly, seven of nine tested genes ($q \leq 0.2$) representing lipid and amino acid metabolic processes in fat and cell proliferation and energy metabolism in liver were validated as DE in LRFI vs. HRFI pigs (Fig. 1*B*). For all tested genes, expression differences were consistent in direction with the microarray results (Fig. 1, *A* and *B*). Statistical significance of effects of CR treatment or RFI ($P < 0.05$) was confirmed by *q*PCR for all genes, except for SLC7A9 ($P = 0.09$) and ITGA6 ($P = 0.2$) in liver of HRFI vs. LRFI pigs (Fig. 1*B*). The results obtained from microarray were statistically confirmed for 88% of the tested genes in liver and fat.

Biological Processes Affected by CR and RFI

To identify biological processes that respond to CR or RFI, lists of upregulated or downregulated DE genes in liver and fat were explored for overrepresentation of GO Biological Process categories. Lists of genes with overrepresented terms were further clustered by similarity of the associated GO term to increase sensitivity. Only pathways overrepresented by the CR-induced DE genes in fat and liver resulted in significant clusters (Enrichment score' 1.3, Supplemental Tables 4, A–D). In addition, the KEGG database was used to identify specific pathways that were overrepresented by genes that responded to RFI or CR treatments ($P \leq 0.1$) in liver or adipose tissues. KEGG annotations that were overrepresented by CR-induced DE genes in fat or liver (Fig. 2) and by RFI-induced genes in fat (Table 3) are summarized and described further in the following sections. The 147 liver genes DE between RFI groups did not result in any significant KEGG term.

Caloric restriction effects in fat. Subcutaneous adipose tissue underwent major transcriptional changes in response to 8-day CR that were reflected by the large number of DE genes (6,114), compared with the corresponding DE genes in liver (305). Caloric restriction caused down-regulation of adipose genes involved in direct energy generation pathways, such as oxidative phosphorylation, TCA cycle, and ATP synthesis. Indicative of metabolic changes that occurred in response to CR, adipose genes in many of the metabolic pathways, including the metabolism of carbohydrate, amino acid, and lipid were down-regulated (Fig. 2; see Supplemental Table 4*A* in the online version of this article). Genes involved in lipid biosynthesis such as steroid and fatty acid synthesis were downregulated, as were genes in degradation of branched chain amino acids and glycolysis. Lipid and steroid pathways were also overrepresented when a list of the 50 DE genes with the highest fold change was analyzed (see Supplemental Table 5 in the online version of this article). Caloric restriction increased expression of genes involved in gene regulation, such as establishment and maintenance of chromatin architecture, DNA packaging, and RNA splicing, as well as genes in several immune response pathways, such as T-cell receptor signaling, natural killer cell-mediated cytotoxicity, complement and coagulation cascades, and the adipocytokine signaling pathway (Fig. 2; Supplemental Table 4*B*).

Caloric restriction effects in liver. In response to the 8-day CR, genes involved in the biosynthesis of macromolecules, such as steroid and protein synthesis were downregulated in liver. Caloric restriction also resulted in downregulation of genes in protein secretion and transport in liver (Fig. 2; Supplemental Table 4*C*). Transport and localization pathways were also overrepresented when a list of the 50 liver genes with the highest fold changes was analyzed (see Supplemental Table 5 in the online version of this article). Caloric restriction also induced upregulation of genes in genetic information processing pathways such as ribosome and proteasome, as well as ion transport processes, namely phosphate transport (Fig. 2, Supplemental Table 4*D*).

Effects due to RFI differences in fat. In LRFI animals, genes involved in lipid metabolic pathways, such as short chain fatty acid and monocarboxylic acid metabolism were downregulated. Genes involved in cellular homeostasis were downregulated in subcutaneous fat of LRFI pigs along with genes in generation of precursor metabolites and energy pathways (Table 3). Compared with HRFI pigs, genes in carbohydrate metabolism were upregulated in adipose tissue of LRFI pigs (Table 3).

Shared Genes and Pathways Between Treatments or Tissues

Commonly shared upregulated or downregulated genes either between liver and adipose tissues within the same treatment or between different treatments within the same tissue were identified (Fig. 3, *A* and *B*). These commonly shared genes were then analyzed for overrepresented GO categories ($P \leq 0.05$) (Fig. 3, *A* and *B*).

Genes and pathways that responded to CR in both fat and liver. Of genes responding to CR in liver, ~29% were also DE in fat. In response to CR, a total of 52 genes were downregulated in both liver and adipose tissues, and they were overrepresented in the steroid and cholesterol biosynthesis

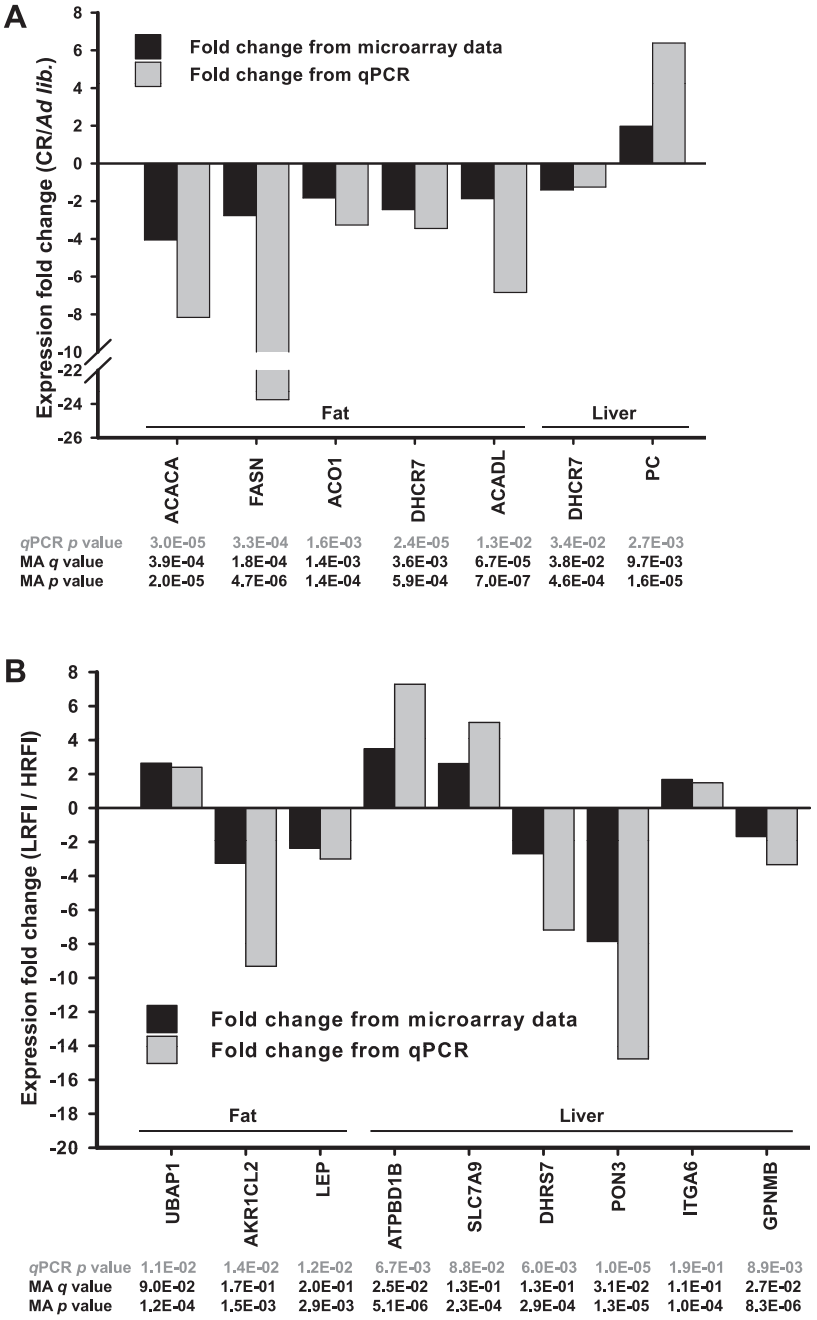

Fig. 1. Microarray data validation by quantitative PCR (qPCR) on a subset of caloric restriction (CR)- or residual feed intake (RFI)-induced differentially expressed (DE) genes in liver and fat. *A:* representative genes in response to CR ($q \leq 0.05$) in lipid biosynthetic pathways in subcutaneous adipose tissue; gluconeogenesis in the liver (q value, positive false discovery rate). *B:* representative genes ($q \leq 0.2$) in lipid and amino acid metabolic processes in fat; cell proliferation and energy metabolism in liver were validated in low residual feed intake (LRFI) vs. high residual feed intake (HRFI) pigs. Official gene symbols were used as abbreviations. ACACA, acetyl-coenzyme A carboxylase alpha; FASN, fatty acid synthase; ACO1, aconitase 1; DHCR7, 7-dehydrocholesterol reductase; ACADL, acyl-coenzyme A dehydrogenase, long chain; PC, pyruvate carboxylase; UBAP1, ubiquitin-associated protein 1; AKR1CL2, aldo-keto reductase family 1, member C-like 2; LEP, leptin; ATPBD1B, ATP binding domain 1 family, member B; SLC7A9, solute carrier family 7 (cationic amino acid transporter, y+ system), member 9; DHRS7, dehydrogenase/reductase (SDR family) member 7C; PON3, paraoxonase 3; ITGA6, integrin, alpha 6; GPNMB, glycoprotein (transmembrane) nmb; MA, microarray. Fold changes are expressed as either CR/ad libitum or LRFI/HRFI, in which negative fold changes indicate down-regulation, while positive fold changes indicate up-regulation of expression due to CR or LRFI.

pathways (Fig. 3*A*). Likewise, 34 genes were upregulated due to CR in both liver and fat, which resulted in overrepresentation of several metabolic pathways, including nucleic acid metabolism (Fig. 3*B*).

Adipose genes and pathways that responded to both CR and RFI. Genes that were upregulated in adipose tissue of LRFI (vs. HRFI) and CR (vs. AL) pigs were overrepresented in cell communication and signal transduction pathways, including transmembrane receptor protein tyrosine kinase signaling (Fig. 3*B*). Many of the pathways overrepresented among downregulated genes in fat of LRFI pigs (vs. HRFI), such as fatty acid metabolism, membrane organization, endocytosis, and generation of precursor metabolites and energy, were also overrepresented among the 74 downregulated adipose genes in both LRFI and CR compared with HRFI and AL, respectively (Figs. 3 and 4). In fact, 42% of all DE adipose genes due to RFI were also DE in adipose as a result of CR, suggesting common pathways are involved in the response to these two factors.

Key transcriptional regulators of response to CR or RFI. Most of the changes in RNA levels that we observed were likely due to changes in levels of transcription. To understand the main transcriptional regulation involved in the response to CR or RFI, genes that were DE in liver and fat were analyzed for their connections to common TF or nuclear receptor regulators by using Pathway Studio 5.0. The analysis of CR-induced DE genes in adipose tissue was limited to genes with fold change equal to or more than two, because of the large number of DE genes. Connections between TF and their targets

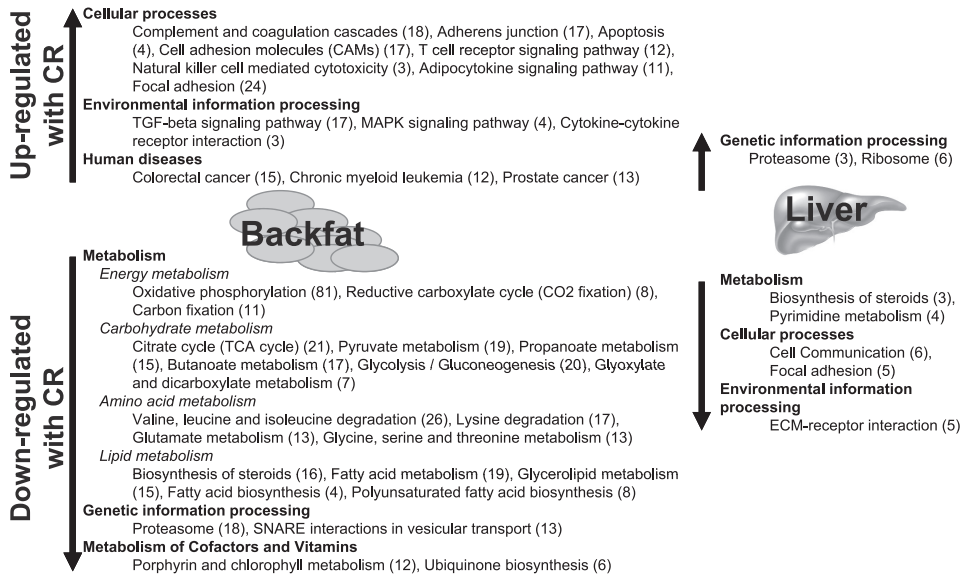

Fig. 2. Functional annotation of liver and adipose tissue-specific response to CR by identifying overrepresented Kyoto Encyclopedia Genes and Genomes (KEGG) biological pathways ($P \leq 0.01$) in differentially expressed genes. Biological processes were subdivided according to KEGG pathway maps.

within the four DE gene lists (i.e., DE genes due to CR or RFI in fat or liver) were populated based on literature evidence of at least one of four interaction categories provided by Pathway Studio 5.0, which were promoter binding, binding, regulation, and direct regulation. Common regulators with the highest number of target genes across all four categories were determined, and their target genes were functionally annotated by GO biological process terms ($P \leq 0.05$) and pathway clusters (Fig. 4).

Table 3. *Overrepresented GO biological processes by the differentially expressed genes in adipose tissue due to RFI differences*

GO ID	Biological Processes (Number of Genes)	*P* Value
	Down-regulated in adipose tissue of LRFI	
32787	monocarboxylic acid metabolic process (8)	4.3E-03
42592	homeostatic process (8)	1.7E-02
48878	chemical homeostasis (6)	2.4E-02
50793	regulation of developmental process (6)	4.7E-02
8535	respiratory chain complex IV assembly (2)	4.8E-02
46459	short-chain fatty acid metabolic process (2)	4.8E-02
6605	protein targeting (6)	4.9E-02
19725	cellular homeostasis (6)	5.0E-02
6811	ion transport (9)	5.8E-02
6631	fatty acid metabolic process (5)	6.0E-02
6091	generation of precursor metabolites and energy (9)	6.7E-02
6897	endocytosis (5)	7.1E-02
10324	membrane invagination (5)	7.1E-02
51052	regulation of DNA metabolic process (3)	7.4E-02
6282	regulation of DNA repair (2)	8.2E-02
16044	membrane organization and biogenesis (6)	8.4E-02
19752	carboxylic acid metabolic process (9)	8.5E-02
6082	organic acid metabolic process (9)	8.6E-02
44255	cellular lipid metabolic process (9)	8.8E-02
7098	centrosome cycle (2)	9.3E-02
725	recombinational repair (2)	9.3E-02
724	double-strand break repair via homologous recombination (2)	9.3E-02
6629	lipid metabolic process (10)	9.9E-02
	Upregulated in adipose tissue of LRFI	
5975	carbohydrate metabolic process (7)	3.5E-02
10468	regulation of gene expression (17)	4.6E-02
6813	potassium ion transport (3)	4.8E-02
6950	response to stress (10)	7.7E-02
10467	gene expression (21)	9.6E-02
44262	cellular carbohydrate metabolic process (5)	9.8E-02

Significance for overrepresented GO biological processes is expressed as $P \leq 0.1$. GO ID, Gene Ontology identification number.

The most connected TF for DE genes in fat due to CR was estrogen receptor 1 (ESR1). This TF had the greatest number of target genes compared with all other identified TF of DE genes in the four categories. Pathway cluster analyses of ESR1 target genes identified four clusters, including homeostasis and cell cycle/apoptosis (Fig. 4). In addition, two transcription factors, HIF1A and Kruppel-like factor 5, were identified as highly connected TF of DE genes with fold changes equal to greater than 2 in fat due to CR.

The most connected TF for the DE genes in the remaining three categories (i.e., liver DE genes due to CR, fat or liver DE genes due to RFI) was transcription factor 1 (SP1). SP1 binds at the GC box promoter elements (41) of target genes, which have a wide array of functions, including embryogenesis, metabolism, growth, vision, and respiratory system (50). As expected, pathway analyses of target genes of SP1 in these three tissue/treatment categories revealed distinct pathways and clusters (Fig. 4). Several of the identified main TF of RFI-induced DE genes in fat or liver were known regulators of energy homeostasis processes, such as PPARG, PPARA, CREB1, and HIF1A.

Key networks of genes involved in the response to CR or RFI differences in fat. Using Pathway Studio 5.0, key gene networks were recognized by counting the number of interactions a DE gene has with other DE genes within each of the four categories of gene lists (i.e., DE genes due to CR or RFI in fat or liver). IGF1 and ESR1 gene networks populated the highest number of interacting genes in response to CR in fat. In response to RFI in fat, the leptin gene network had the highest number of interacting genes (data not shown).

DISCUSSION

Our main findings demonstrate specific transcriptional responses to CR and RFI across liver and adipose tissues with

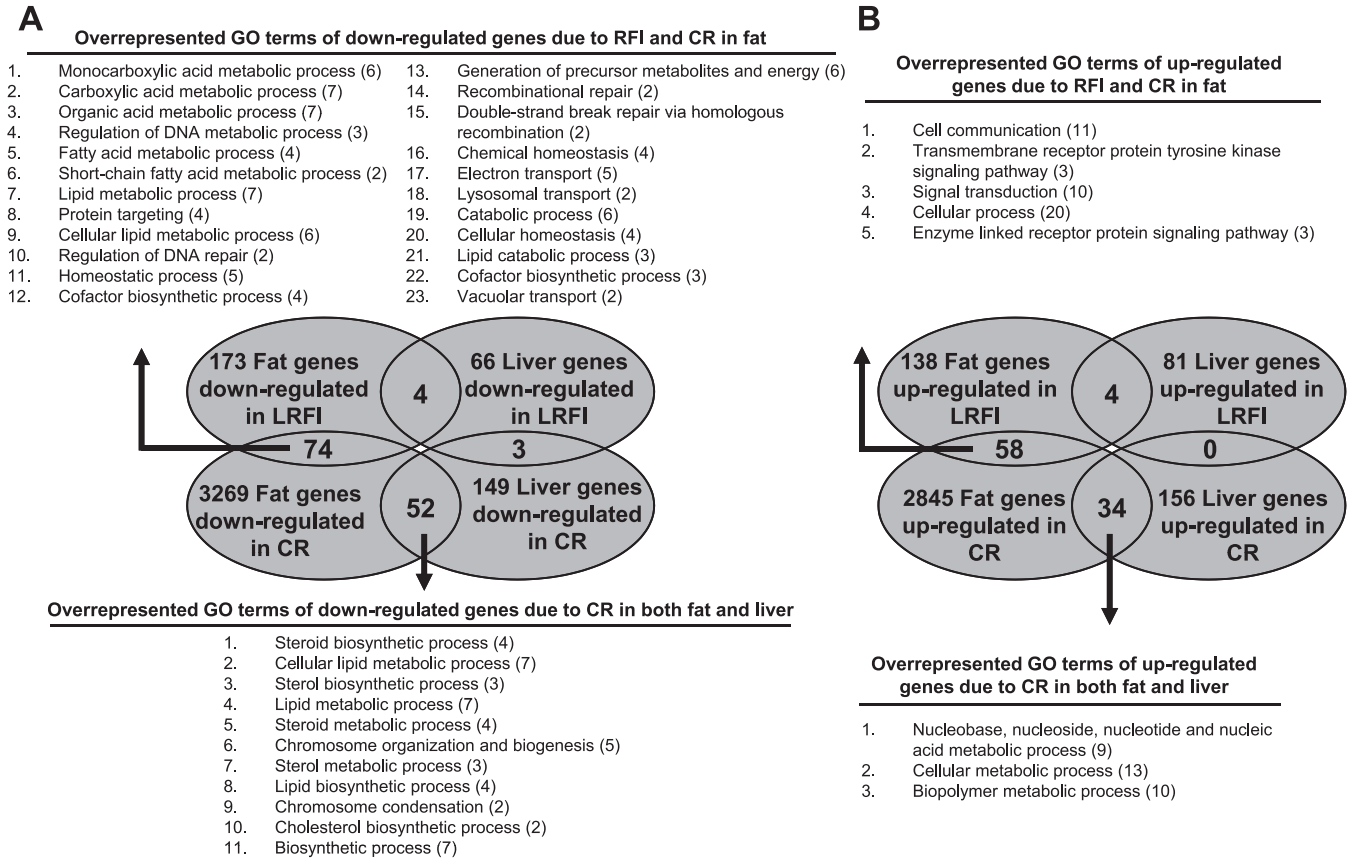

Fig. 3. Shared genes and pathways between fat and liver tissue response to RFI or CR and tissue-specific overlapped genes and pathways between RFI and CR treatments. *A:* overlapment of down-regulated genes and corresponding pathways (GO, $P < 0.05$) *B:* overlapment of up-regulated genes and corresponding pathways (GO, $P < 0.05$).

congruent changes in serum metabolites, and we document metabolic pathways and transcription factors that appear to govern these responses. Although alterations in RNA expression do not always correspond to alterations in protein expression or activity, we suggest that systemic changes in the expression of genes that belong to overrepresented GO or KEGG categories imply functional change of pathways represented by these categories. Statistical significance of genes discussed hereafter is summarized in either Table 4 or Table 5 unless referenced elsewhere.

Steroid biosynthesis pathway was downregulated in both liver and adipose tissues due to CR. As was observed with a CR study in rodents (73), in both liver and adipose tissues, we observed the downregulation of genes in lipid and steroid biosynthetic processes. Of the four genes involved in steroid synthesis that were down-regulated in both fat and liver, the expression of one, 7-dehydrocholesterol reductase (DHCR7), was tested and confirmed by qPCR (Fig. 1*A*). Similar to a related 3-day fasting study of pigs, in which genes with some of the highest fold changes in both fat and liver tissues overrepresented steroid synthesis pathway (52), our results indicated that the 50 DE genes in fat with highest fold changes in response to 8-day CR overrepresented the steroid synthesis pathway.

Caloric restriction induced genes in the immune response pathways. Reports indicate that short term acute CR induces transcriptional changes involved in aging mechanisms in rodents (24, 73). Calorie restriction used in our study was ~25% of AL feed and is comparable to the CR used in longevity studies (38).

A loss of the capacity to remodel neuroendocrine-immune response leads to increased age-associated pathologies (62). Enhancement of immune response may underlie some of the antiaging mechanisms associated with CR (25). We report that 172 genes in immune response pathways were upregulated in response to CR in adipose tissue, which resulted in overrepresented KEGG immune response pathways (Fig. 2) and significantly clustered immune response GO terms (Supplemental Table 4*B*). Therefore, our results indicate that some of the CR-mediated longevity promoting effects may be explained by a stimulation of immune response pathways.

Enhancement of anticoagulatory pathway in response to CR. A correlation between aging and heightened coagulation activity has been documented in humans (43, 55). Plasmin has an anticoagulatory role, in which it degrades many blood plasma proteins and is involved in fibronolysis. Plasminogen (PLG) is a circulating zymogen that is converted to the active enzyme plasmin by cleavage that is mediated by tissue plasminogen activator and urokinase. In our study, PLG was 3.4 fold upregulated as a result of CR along with the gene encoding the tissue plasminogen activator protein, which was 1.5-fold upregulated in the fat due to CR (Table 4). Interestingly, mice overexpressing plasminogen activator have reduced food consumption, body weight, and increased longevity (61). In addi-

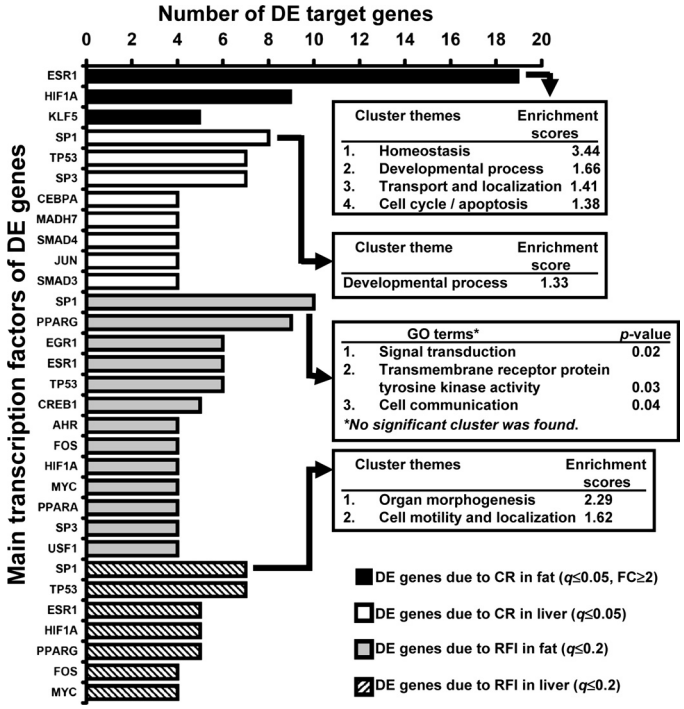

Fig. 4. Transcription factors (TF) with greatest number of connections for responses to CR and RFI in liver and fat and the pathways overrepresented (GO, $P \leq 0.05$) by the target genes of the most connected TF in each gene list. Official gene symbols for the TF and nuclear receptors were used as abbreviations. AHR, aryl-hydrocarbon receptor; CEBPA, CCAAT/enhancer binding protein (C/EBP), alpha; CREB1, cAMP responsive element binding protein 1; EGR1, early growth response 1; ESR1, estrogen receptor 1 (alpha); FOS, FBJ osteosarcoma oncogene; HIF1A, hypoxia inducible factor 1, alpha subunit; JUN, Jun oncogene; KLF5, Kruppel-like factor 5; MADH7, MAD homolog 7; MYC, myelocytomatosis oncogene; PPARA, peroxisome proliferator activated receptor alpha; PPARG, peroxisome proliferator activated receptor gamma; SMAD3, MAD homolog 3; SMAD4, MAD homolog 4; SP1, trans-acting transcription factor 1; SP3, trans-acting transcription factor 3; TP53, transformation related protein 53; USF1, upstream transcription factor 1.

tion, several anticoagulatory modulators such as thrombomodulin and fletcher factor 1 (kallikrein b) were upregulated by CR in our study (Table 4). Thrombin is a serine protease that has many catalytic effects in the coagulation cascade, including converting soluble fibrinogen into insoluble strands of fibrin (48). Thrombomodulin, which was 1.5-fold upregulated by CR in our study (Table 4), forms a complex with thrombin and functions as an anticoagulant factor in the thrombin-induced activation of protein C (23). Kallikrein b is a serine protease involved in the kinin-kallilkrein system, which plays a role in blood pressure control, coagulation, inflammation, and pain. Human kallikrein gene delivery has been shown to attenuate hypertension (14), and although no direct relation between aging and kallikrein has been established, genetic studies have indicated a correlation between longevity and cardiovascular risk markers, such as apolipoprotein E and angiotensin-converting enzyme (4, 7). Taken together, our data indicate that CR enhances anticoagulatory mechanisms in the pig, which may contribute to longevity-promoting pathways that are associated with CR.

T cell receptor signaling pathway in CR. Adipose tissue contains diverse cell types, including T cells, macrophages, fibroblasts, endothelial cells, and multipotent mesenchymal cells according to studies in rodents (26, 36). T cells play a central role in cell-mediated immunity and T cell receptor (TCR) activation, and they promote a number of signaling cascades that determine cell fate by regulating cytokine production, cell survival, proliferation, and differentiation (10). Aging leads to an accumulation of T cells with signal transduction defects (70), and such changes in T lymphocyte populations underlie much of the age-related decline in the protective immune response (60). In our study, 13 genes that were upregulated with CR were annotated as having direct involvement in the T cell receptor signaling pathway (Fig. 2, Table 4). Of these, several genes such as nonreceptor type 6 protein tyrosine phosphatase (PTPN6), E3 ubiquitin ligase casitas B-lineage lymphoma-b (CBLB), and tec protein tyrosine kinase, are involved in activation of TCR signaling. PTPN6 expression was upregulated 3.7 fold in fat in response to CR in our study. The N-terminal part of PTPN6 contains two tandem *src* homolog domains, which act as protein phosphotyrosine binding domains and mediate the interaction of protein tyrosine phosphatase with its substrates in TCR signaling (88). Z-chain-associated protein kinase (ZAP-70) is recruited to the TCR/CD3 complex and activates the T cell (65). PTPN6 can bind to ZAP-70, resulting in suppression of T-cell activation by increasing PTPN6 phosphatase activity, thus decreasing ZAP-70 kinase activity (65). Another protein that negatively regulates ZAP-70 is CBLB, and it was 1.4 fold upregulated with CR in our study. CBLB plays an important role in regulating the threshold of signaling in T cells and knockouts of CBLB results in an increased susceptibility to development of autoimmunity (49).

Results in our study indicate that some genes that have a stimulating effect on the TCR activation were also upregulated with CR. Activation of Src-family kinase FYN is central to the initiation of TCR signaling pathways (71) and was 1.3 fold upregulated by CR in our study. Our results also show that processes downstream of TCR activation, including MAPK, NF-κB, and Ca^{2+} signaling, are affected by CR. Genes in the MAPK signaling pathway, such as MAPK8, growth factor receptor-bound protein 2, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, and genes in Ca^{2+} signaling, such as nuclear factor of activated T-cells 5 and protein phosphatase 3 catalytic subunit, beta isoform, were upregulated in response to CR. The NF-κB complex is inhibited by I-κB proteins (e.g., NFKBIA), which inactivate NF-κB by trapping it in the cytoplasm. In our study, NFKBIA was also 1.6 fold upregulated due to CR. T cell markers, such as CD4 and CD8, were not differentially expressed in our study, indicating that the changes in expression of genes in TCR signaling were likely transcriptional rather than indicating change in cell type frequencies in these tissues (Table 4). Taken together, our results indicate that CR modulated TCR signaling via changes in expression of genes involved in both activation of TCR signaling and its downstream effectors.

ESR1 Targets CR-Induced DE Genes in Cell Cycle and Apoptotic Pathways

Transcription factors play key roles in regulating adaptive processes involved in energy restriction. We identified several TF that were previously known to be involved in energy homeostatic processes, such as CREB1, HIF1A, PPARG, and

Table 4. *Statistical significance of genes involved in the CR induced immune response and cell cycle/apoptosis pathways in subcutaneous adipose tissue*

Pathways	Gene Description	GenBank Symbol	Affymetrix ID	*Fold Change	*q* Value	*P* Value
Anticoagulation	plasminogen	PLG	Ssc.7237	3.36	3.4E-02	1.4E-02
	plasminogen activator, tissue	PLAT	Ssc.196	1.53	1.3E-02	3.7E-03
	thrombomodulin	THBD	Ssc.20711	1.50	4.0E-02	1.7E-02
	kallikrein b, plasma (fletcher factor) 1	KLKB1	Ssc.260	2.24	4.1E-03	7.0E-04
	thrombospondin 1	THBS1	Ssc.26702	2.81	4.6E-02	2.1E-02
T-cell receptor signaling	protein tyrosine phosphatase; nonreceptor type 6	PTPN6	Ssc.5530	3.74	1.9E-02	6.3E-03
	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; alpha	NFKBIA	Ssc.4759	1.64	8.3E-04	6.3E-05
	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	Ssc.30766	1.55	8.8E-04	7.0E-05
	tumor necrosis factor (TNF superfamily; member 2)	TNF	Ssc.100	1.61	2.4E-02	8.4E-03
	mitogen-activated protein kinase kinase kinase 8	MAP3K8	Ssc.18038	1.55	4.7E-03	8.5E-04
	tec protein tyrosine kinase	TEC	Ssc.4466	1.43	1.3E-02	3.7E-03
	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	CBLB	Ssc.2897	1.38	1.7E-02	5.3E-03
	FYN oncogene related to SRC	FYN	Ssc.2714	1.25	4.9E-02	2.4E-02
	nuclear factor of activated T-cells 5; tonicity-responsive	NFAT5	Ssc.17745	1.25	5.3E-03	9.7E-04
	Cas-Br-M ecotropic retroviral transforming sequence	CBL	Ssc.24231	1.22	5.8E-03	1.1E-03
	growth factor receptor-bound protein 2	GRB2	Ssc.27313	1.21	1.2E-02	3.0E-03
	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	KRAS	Ssc.2013	1.19	3.3E-02	1.3E-02
	protein phosphatase 3 (formerly 2B); catalytic subunit; beta isoform	PPP3CB	Ssc.22111	1.12	3.3E-02	1.3E-02
T-cell markers	CD8a molecule	CD8A	Ssc.23489	1.09	3.5E-01	4.7E-01
	CD8b molecule	CD8B	Ssc.23495	1.1	3.7E-01	5.3E-01
	CD4 molecule	CD4	Ssc.16240	1.73	1.3E-01	1.0E-01
Cell cycle/apoptosis	cyclin-dependent kinase inhibitor 1A	CDKN1A	Ssc.5737	−2.33	2.4E-04	7.7E-06
	breast cancer 1; early onset	BRCA1	Ssc.24110	−2.40	3.5E-02	1.4E-02
	glial cell line derived neurotrophic factor	GDNF	Ssc.21655	2.36	1.2E-02	3.1E-03
	nonmetastatic cells 1	NME1	Ssc.19546	−2.10	1.4E-04	2.8E-06
	Sus scrofa tumor necrosis factor (ligand) superfamily; member 10	TNFSF10	Ssc.12829	1.92	1.5E-03	1.5E-04
	phosphoinositide-3-kinase; regulatory subunit 5	PIK3R5	Ssc.11020	1.53	1.7E-01	1.4E-01
	TNF receptor superfamily; member 6	FAS	Ssc.11440	1.50	2.7E-02	1.0E-02
	Fas-associated via death domain	FADD	Ssc.1947	1.39	1.2E-03	1.1E-04

*Fold changes are expressed as caloric restriction (CR)/ad libitum when CR > ad libitum (upregulation of expression due to CR) and the negative of ad libitum/CR when CR < ad libitum (downregulation of expression due to CR). *q* Value, positive false discovery rate.

PPARA (22), as well as several TF not previously implicated in these processes. The role of ESR1 in adaptation to feed restriction is not documented. However, an emerging importance of ESR1 in metabolic systems is evident, as a number of ESR1 polymorphisms have been associated with metabolic syndromes like atherosclerosis and type II diabetes (19, 29, 37, 58). Moreover, in hepatic tissue, administration of estradiol downregulates expression of lipogenic genes and improves insulin sensitivity (30). ESR1 was 2.4-fold upregulated with CR ($q = 0.03$, $P = 0.01$) and had the highest number of DE target genes compared with any other TF in the four categories of genes in our study (Fig. 4). In a related 3-day fasting study in pigs, we found ESR1 to be significantly upregulated and to target many of the DE genes in fat due to fasting (52). In the current study, 19 CR-induced DE genes in adipose tissue have been shown in the literature of being directly regulated by

Table 5. *Statistical significance of CR- and RFI-induced genes in lipid metabolism pathway*

GenBank Symbol	Gene Description	Affymetrix ID	RFI *q* Value	RFI *P* Value	RFI Fold Change*	CR *q* Value	CR *P* Value	CR Fold Change**
NPC1	Niemann-Pick disease; type C1	Ssc.1533	1.8E-02	2.5E-06	−1.41	5.4E-04	3.3E-05	−1.31
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	Ssc.29842	4.7E-02	1.7E-05	−1.60	1.7E-04	4.3E-06	−1.69
SMPD1	sphingomyelin phosphodiesterase 1	Ssc.3927	1.9E-01	2.3E-03	−1.18	3.7E-05	1.7E-07	−1.55
ACADSB	acyl-coenzyme A dehydrogenase; short/branched chain	Ssc.7572	2.0E-01	2.6E-03	−1.46	5.1E-04	3.0E-05	−1.88
GCLM	glutamate-cysteine ligase; modifier subunit	Ssc.24693	1.1E-01	2.5E-04	−1.36	1.0E-04	1.6E-06	−1.66
LIPG	lipase; endothelial	Ssc.21663	1.8E-01	1.8E-03	−2.02	2.6E-02	9.3E-03	−1.73
BRCA1	breast cancer 1; early onset	Ssc.29471	2.0E-01	2.4E-03	−1.40	4.1E-03	7.0E-04	−1.50
OXSM	3-oxoacyl-ACP synthase; mitochondrial	Ssc.11586	1.6E-01	1.0E-03	−1.28	2.9E-03	4.2E-04	−1.32
ALDH5A1	aldehyde dehydrogenase 5 family; member A1	Ssc.27232	1.8E-01	1.7E-03	−1.43	3.2E-02	1.2E-02	−1.30

*RFI fold changes are expressed as LRFI/HRFI when LRFI > HRFI (upregulation of expression in LRFI) and negative of HRFI/LRFI when HRFI > LRFI (downregulation of expression due in LRFI). **CR fold changes are expressed as as CR/ad libitum when CR > ad libitum (upregulation of expression due to CR) and the negative of ad libitum/CR when CR < ad libitum (downregulation of expression due to CR).

ESR1, and these genes belonged to pathways of energy homeostasis, development, protein transport, and localization, and apoptosis (Fig. 4).

The lifespan of a cell depends on the balance between prosurvival and apoptotic or death-promoting factors. Longevity-promoting effects that are associated with CR have been attributed to changes in apoptotic and cell survival pathways in many cell types, including neurons, renal cells, and hepatocytes (5, 17, 78, 87). In our study, several pathways involved in apoptosis and cell survival/proliferation were overrepresented by the CR-induced upregulated fat genes, such as Fas receptor, Fas-associated death domain-containing protein, p101 protein, and NF-κB1 (Fig. 2, Table 4, Supplemental Table 4*A*). Interestingly, target genes of ESR1, which were more than twofold DE in adipose tissue in response to CR, overrepresented pathways that pertain to cell cycle, cell proliferation, and cell death, such as cyclin-dependent kinase inhibitor, breast cancer 1, early onset, nonmetastatic cells 1 genes (Table 4). Fat cell number increases with age and restriction of energy intake reduces the number of fat cells in fat depots (56, 75). In our study, loss of backfat depth along with a trend of increased nonesterified fatty acids in the bloodstream associated with CR was observed. We also confirmed the expression of CR-induced downregulation of five genes in fat that are involved in lipogenesis (*ACACA*, *FASN*, *ACO1*, *DHCR7*, *ACADL*) by qPCR (Fig. 1*A*). Therefore, the upregulated expression of proapoptotic genes in fat due to CR may explain the previously documented CR-induced decrease in number and size of adipocytes (56, 75). Our in silico literature-based transcriptional factor analyses also indicate that CR-induced genes in apoptotic pathway are regulated by ESR1. Taken together, we propose that the increase in lifespan associated with CR may be attributable to changes in expression of genes in apoptotic pathways that, in part, may be under the transcriptional control of ESR1.

Transcriptional Differences due to RFI Correspond to Lipid Metabolism

We report the transcriptional response of many genes in fat and liver that have not been previously reported to be associated with feed efficiency or RFI. A difference in lipid metabolism in the adipose tissue between RFI groups was not unexpected because a previous study on selection response for RFI in Yorkshire swine found a 10th rib backfat depth of LRFI pigs was 2.62 ± 1.01 mm ($P < 0.05$) less than the control pigs, which were the basis of the current study (12). This was comparable to findings in the present study that 10th rib backfat of LRFI pigs was thinner than that of HRFI pigs (Table 1). Expectedly, the transcriptional response to RFI differences resulted in downregulation of genes that overrepresented many pathways involved in lipid metabolic processes, such as carboxylic acid, monocarboxylic acid, fatty acid, and short-chain fatty acid metabolism (Table 3). Our results on RFI-induced transcriptional changes of genes in lipid metabolism explain some of the previously reported differences in fat content of poultry (27, 54) and cattle (3, 35) with divergent RFI. Serum leptin concentration has been shown to positively correlate with RFI values in pigs and cattle (35, 64). In our study, serum leptin concentration did not differ between the two lines, and this is likely attributable to the limited number of animals used in this experiment. On the other hand, we report that expression of leptin was 2.4-fold downregulated in LRFI pigs, and this was confirmed by qPCR (Fig. 1*B*). Therefore, abundance of leptin transcripts may be a more sensitive marker to evaluate feed efficiency-associated traits compared with the serum leptin concentration. Key gene network analyses in our study indicated that leptin had the greatest number of interacting DE genes due to RFI differences, suggesting that the leptin gene network is a potentially important component contributing to RFI differences.

Interestingly, our intertreatment analyses found 74 downregulated genes in fat due to both CR response and RFI differences, and these genes overrepresented the same lipid metabolic pathways that were overrepresented by adipose genes induced by RFI differences alone (Fig. 3, Table 3). The CR- and RFI-induced downregulated genes in lipid metabolic processes are summarized in Table 5. This indicates that the lipid metabolic processes in feed-efficient LRFI animals were altered, compared with HRFI pigs, in a similar fashion as in the CR-treated animals and may explain previously reported CR-driven feed efficiency (67, 69). Changes in expression of lipid metabolic genes are likely attributable to the feed efficiency observed in LRFI pigs.

Our TF analyses revealed 13 TF that are known to regulate genes found to be DE as a result of RFI differences in adipose tissue. Some of these TF, such as PPARG, PPARA, and CREB1, target genes that belong to lipid metabolic processes. For example, a target of PPARA, endothelial cell-derived lipase (LIPG) has phospholipase and triglyceride lipase activities and hydrolyzes high-density lipoproteins more efficiently than other lipoproteins (39, 40). Overexpression of LIPG in mice reduces plasma concentration of HDL cholesterol (40) and LIPG was downregulated by 2.0- and 1.7-fold due to RFI and CR, respectively (Table 5). Another target of PPARA is the Niemann-Pick disease type C1 (NPC1) gene, which encodes a putative integral membrane protein and plays a role in intracellular transport of cholesterol to postlysosomal destinations (53). The NPC1 RNA was 1.4- and 1.3-fold downregulated in CR treated pigs (vs. AL) and LRFI pigs (vs. HRFI), respectively. Mitochondrial glycerophosphate dehydrogenase (GPD2) was one of the RFI-induced genes targeted by both CREB1 and PPARG (Fig. 4). The protein encoded by GPD2 is located on the outer surface of the inner mitochondrial membrane and catalyzes the unidirectional conversion of glycerol-3-phosphate to dihydroxyacetone phosphate, which eventually feeds into glycolysis (32). GPD2 was downregulated 1.6-fold in LRFI and 1.7-fold in CR animals in our study. Such commonality in transcriptional response between the RFI and CR-associated pathways indicate that LRFI pigs may mimic the energy conservation process seen in response to CR in the adipose tissue via downregulation of genes in lipid metabolism. Moreover, some of the RFI-induced lipid metabolic genes in adipose tissue have been reported to be transcriptionally controlled by PPARA, PPARG, and CREB1. Perhaps the differential regulation of lipid metabolic processes can explain some of the feed efficiency differences seen between LRFI and HRFI pigs.

Triiodothyronine and RFI. Serum triiodothyronine has been associated with positive growth traits during early postnatal development of several species, including cattle and swine (8, 13, 34, 46). However, conflicting results have been reported

regarding the association of triiodothyronine concentration with RFI during the growing phase of the animal (27, 28). In our study, serum concentration of total triiodothyronine was higher in LRFI than HRFI pigs (Table 2). Thyroid hormones affect multiple processes in all organ systems; therefore, to elucidate the connection between RFI and triiodothyronine in pigs, further studies are needed to evaluate the biology of the thyroid gland in reference to RFI differences.

Perspectives and Significance

This is the first study to report transcriptional changes associated with both RFI differences and response to CR. We report that genes in lipid metabolic processes were significantly altered in adipose tissue due to RFI and CR, which suggests that LRFI pig shifted to energy conservation and efficient utilization pathways. These CR- and RFI-induced regulatory changes may be controlled by PPARA, PPARG, and CREB1. Caloric restriction modulated immune pathways, which may explain, in part, CR-driven lifespan enhancement. We further provide evidence for the role of ESR1 in the adipose tissue in response to short-term acute CR.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

1. **Affymetrix.** Statistical algorithms description document. http://www.affymetrix.com/support/technical/whitepapers/sadd_whitepaper.pdf, 2002.
2. **Albanes D.** Caloric intake body weight, cancer: a review. *Nutr Cancer* 9: 199–217, 1987.
3. **Arthur PF, Archer JA, Johnston DJ, Herd RM, Richardson EC, Parnell PF.** Genetic and phenotypic variance and covariance components for feed intake, feed efficiency, and other postweaning traits in Angus cattle. *J Anim Sci* 79: 2805–2811, 2001.
4. **Atzmon G, Rincon M, Schechter CB, Shuldiner AR, Lipton RB, Bergman A, Barzilai N.** Lipoprotein genotype and conserved pathway for exceptional longevity in humans. *PLoS Biol* 4: e113, 2006.
5. **Balan V, Miller GS, Kaplun L, Balan K, Chong ZZ, Li F, Kaplun A, VanBerkum MF, Arking R, Freeman DC, Maiese K, Tzivion G.** Life span extension and neuronal cell protection by *Drosophila* nicotinamidase. *J Biol Chem* 283: 27810–27819, 2008.
6. **Barendse W, Reverter A, Bunch RJ, Harrison BE, Barris W, Thomas MB.** A validated whole-genome association study of efficient food conversion in cattle. *Genetics* 176: 1893–1905, 2007.
7. **Baudin B.** Angiotensin I-converting enzyme gene polymorphism and drug response. *Clin Chem Lab Med* 38: 853–856, 2000.
8. **Beaver EE, Williams JE, Miller SJ, Hancock DL, Hannah SM, O'Connor DL.** Influence of breed and diet on growth, nutrient digestibility, body composition and plasma hormones of Brangus and Angus steers. *J Anim Sci* 67: 2415–2425, 1989.
9. **Bottje WG, Carstens GE.** Association of mitochondrial function and feed efficiency in poultry and livestock species. *J Anim Sci* 87: E48–E63, 2009.
10. **Burbach BJ, Medeiros RB, Mueller KL, Shimizu Y.** T-cell receptor signaling to integrins. *Immunol Rev* 218: 65–81, 2007.
11. **Bustin SA.** Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 29: 23–39, 2002.
12. **Cai W, Casey DS, Dekkers JC.** Selection response and genetic parameters for residual feed intake in Yorkshire swine. *J Anim Sci* 86: 287–298, 2008.
13. **Cartwright AL, Leatherwood JM, Eisen EJ.** Thyroid hormones and efficiency of energy utilization in mice selected for body weight. *J Nutr* 110: 1262–1273, 1980.
14. **Chao J, Zhang JJ, Lin KF, Chao L.** Human kallikrein gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in Dahl salt-sensitive rats. *Hum Gene Ther* 9: 21–31, 1998.
15. **Chapin RB, Brady PS, Barke RA, Brady LJ.** Hepatic CCAAT/enhancer binding protein (C/EBP-α and C/EBP-β) expression changes with riboflavin deficiency, diet restriction and starvation in rats. *J Nutr* 124: 2365–2375, 1994.
16. **Chen Y, Gondro K, Quinn K, Herd R.** Gene expression for cattle with high or low feed efficiency (Abstract). *Proceedings from the International Society of Animal Genetics.* Hoboken, NJ: Wiley, 2008.
17. **Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA.** Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305: 390–392, 2004.
18. **Couture O, Callenberg K, Kaul N, Pandit S, Younes R, Hu Z, Dekkers J, Reecy J, Honavar V, Tuggle C.** ANEXdb: an integrated animal ANnotation and microarray EXpression database. *Mamm Genome*. In Press.
19. **Dahlman I, Vaxillaire M, Nilsson M, Lecoeur C, Gu HF, Cavalcanti-Proenca C, Efendic S, Ostenson CG, Brismar K, Charpentier G, Gustafsson JA, Froguel P, Dahlman-Wright K, Steffensen KR.** Estrogen receptor alpha gene variants associate with type 2 diabetes and fasting plasma glucose. *Pharmacogenet Genomics* 18: 967–975, 2008.
20. **Dawson HD, Beshah E, Nishi S, Solano-Aguilar G, Morimoto M, Zhao A, Madden KB, Ledbetter TK, Dubey JP, Shea-Donohue T, Lunney JK, Urban JF Jr.** Localized multigene expression patterns support an evolving Th1/Th2-like paradigm in response to infections with *Toxoplasma gondii* and *Ascaris suum*. *Infect Immun* 73: 1116–1128, 2005.
21. **Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, and Lempicki RA.** DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4: P3, 2003.
22. **Desvergne B, Michalik L, Wahli W.** Transcriptional regulation of metabolism. *Physiol Rev* 86: 465–514, 2006.
23. **Esmon CT.** Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *FASEB J* 9: 946–955, 1995.
24. **Estep PW, 3rd Warner JB, Bulyk ML.** Short-term calorie restriction in male mice feminizes gene expression and alters key regulators of conserved aging regulatory pathways. *PloS One* 4: e5242, 2009.
25. **Fernandes G.** Progress in nutritional immunology. *Immunol Res* 40: 244–261, 2008.
26. **Ferrante AW Jr.** Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. *J Intern Med* 262: 408–414, 2007.
27. **Gabarrou JF, Geraert PA, Picard M, Bordas A.** Diet-induced thermogenesis in cockerels is modulated by genetic selection for high or low residual feed intake. *J Nutr* 127: 2371–2376, 1997.
28. **Gabarrou JF, Geraert PA, Williams J, Ruffier L, Rideau N.** Glucose-insulin relationships and thyroid status of cockerels selected for high or low residual food consumption. *Br J Nutr* 83: 645–651, 2000.
29. **Gallagher CJ, Langefeld CD, Gordon CJ, Campbell JK, Mychaleckyj JC, Bryer-Ash M, Rich SS, Bowden DW, Sale MM.** Association of the estrogen receptor-alpha gene with the metabolic syndrome and its component traits in African-American families: the Insulin Resistance Atherosclerosis Family Study. *Diabetes* 56: 2135–2141, 2007.
30. **Gao H, Bryzgalova G, Hedman E, Khan A, Efendic S, Gustafsson JA, Dahlman-Wright K.** Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in *ob/ob* mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. *Mol Endocrinol* 20: 1287–1299, 2006.
31. **Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J.** Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5: R80, 2004.
32. **Gerbitz KD, Gempel K, Brdiczka D.** Mitochondria and diabetes. Genetic, biochemical, and clinical implications of the cellular energy circuit. *Diabetes* 45: 113–126, 1996.
33. **Graeme T, Roese G.** Pork-cost of production. *New South Wales Department of Primary Industries* 66: 1–3, 2006.

34. **Harrison AP, Tivey DR, Clausen T, Duchamp C, Dauncey MJ.** Role of thyroid hormones in early postnatal development of skeletal muscle and its implications for undernutrition. *Br J Nutr* 76: 841–855, 1996.
35. **Hoque MA, Katoh K, Suzuki K.** Genetic associations of residual feed intake with serum IGF-I and leptin concentrations, meat quality, and carcass cross sectional fat area ratios in Duroc pigs. *J Anim Sci* 87: 3069–3075, 2009.
36. **Hotamisligil GS.** Inflammation and metabolic disorders. *Nature* 444: 860–867, 2006.
37. **Huang Q, Wang TH, Lu WS, Mu PW, Yang YF, Liang WW, Li CX, Lin GP.** Estrogen receptor alpha gene polymorphism associated with type 2 diabetes mellitus and the serum lipid concentration in Chinese women in Guangzhou. *Chin Med J (Engl)* 119: 1794–1801, 2006.
38. **Hursting SD, Lavigne JA, Berrigan D, Perkins SN, Barrett JC.** Calorie restriction, aging, and cancer prevention: mechanisms of action and applicability to humans. *Annu Rev Med* 54: 131–152, 2003.
39. **Ishida T, Choi S, Kundu RK, Hirata K, Rubin EM, Cooper AD, Quertermous T.** Endothelial lipase is a major determinant of HDL level. *J Clin Invest* 111: 347–355, 2003.
40. **Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, South V, Amin D, Perrone M, Rader DJ.** A novel endothelial-derived lipase that modulates HDL metabolism. *Nat Genet* 21: 424–428, 1999.
41. **Kadonaga JT, Carner KR, Masiarz FR, Tjian R.** Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* 51: 1079–1090, 1987.
42. **Kang MJ, Kim HJ, Kim HK, Lee JY, Kim DH, Jung KJ, Kim KW, Baik HS, Yoo MA, Yu BP, Chung HY.** The effect of age and calorie restriction on HIF-1-responsive genes in aged liver. *Biogerontology* 6: 27–37, 2005.
43. **Kannel WB, Wolf PA, Castelli WP, D'Agostino RB.** Fibrinogen and risk of cardiovascular disease. The Framingham Study. *JAMA* 258: 1183–1186, 1987.
44. **Kenward MG, Roger JH.** Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* 53: 983–997, 1997.
45. **Kim KS, Larsen N, Short T, Plastow G, Rothschild MF.** A missense variant of the porcine melanocortin-4 receptor (MC4R) gene is associated with fatness, growth, and feed intake traits. *Mamm Genome* 11: 131–135, 2000.
46. **King DB, May JD.** Thyroidal influence on body growth. *J Exp Zool* 232: 453–460, 1984.
47. **Koch RM, Siger LA, Chambers D, Gregory KE.** Efficiency of feed use in beef cattle. *J Anim Sci* 22: 486–494, 1963.
48. **Lenting PJ, van Mourik JA, Mertens K.** The life cycle of coagulation factor VIII in view of its structure and function. *Blood* 92: 3983–3996, 1998.
49. **Li D, Gal I, Vermes C, Alegre ML, Chong AS, Chen L, Shao Q, Adarichev V, Xu X, Koreny T, Mikecz K, Finnegan A, Glant TT, Zhang J.** Cutting edge: Cbl-b: one of the key molecules tuning CD28- and CTLA-4-mediated T cell costimulation. *J Immunol* 173: 7135–7139, 2004.
50. **Li L, He S, Sun JM, Davie JR.** Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* 82: 460–471, 2004.
51. **Liu WM, Mei R, Di X, Ryder TB, Hubbell E, Dee S, Webster TA, Harrington CA, Ho MH, Baid J, Smeekens SP.** Analysis of high-density expression microarrays with signed-rank call algorithms. *Bioinformatics* 18: 1593–1599, 2002.
52. **Lkhagvadorj S, Qu L, Cai W, Couture OP, Barb CR, Hausman GJ, Nettleton D, Anderson LL, Dekkers JC, Tuggle CK.** Microarray gene expression profiles of fasting induced changes in liver and adipose tissues of pigs expressing the melanocortin-4 receptor D298N variant. *Physiol Genomics* 38: 98–111, 2009.
53. **Loftus SK, Morris JA, Carstea ED, Gu JZ, Cummings C, Brown A, Ellison J, Ohno K, Rosenfeld MA, Tagle DA, Pentchev PG, Pavan WJ.** Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* 277: 232–235, 1997.
54. **Luiting P, Urff EM.** Residual feed consumption in laying hens. 2. Genetic variation and correlations. *Poult Sci* 70: 1663–1672, 1991.
55. **Mari D, Coppola R, Provenzano R.** Hemostasis factors and aging. *Exp Gerontol* 43: 66–73, 2008.
56. **Masoro EJ.** Aging and proliferative homeostasis: modulation by food restriction in rodents. *Lab Anim Sci* 42: 132–137, 1992.
57. **Masternak MM, Al-Regaiey KA, Del Rosario Lim MM, Jimenez-Ortega V, Panici JA, Bonkowski MS, Kopchick JJ, Bartke A.** Effects of caloric restriction and growth hormone resistance on the expression level of peroxisome proliferator-activated receptors superfamily in liver of normal and long-lived growth hormone receptor/binding protein knockout mice. *J Gerontol A Biol Sci Med Sci* 60: 1394–1398, 2005.
58. **Mattsson C, Olsson T.** Estrogens and glucocorticoid hormones in adipose tissue metabolism. *Curr Med Chem* 14: 2918–2924, 2007.
59. **Miller ER, Ullrey DE.** The pig as a model for human nutrition. *Annu Rev Nutr* 7: 361–382, 1987.
60. **Miller RA.** The aging immune system: primer and prospectus. *Science* 273: 70–74, 1996.
61. **Miskin R, Masos T.** Transgenic mice overexpressing urokinase-type plasminogen activator in the brain exhibit reduced food consumption, body weight and size, and increased longevity. *J Gerontol A Biol Sci Med Sci* 52: B118–B124, 1997.
62. **Mocchegiani E, Malavolta M.** Possible new antiaging strategies related to neuroendocrine-immune interactions. *Neuroimmunomodulation* 15: 344–350, 2008.

62a. **National Research Council** *Nutrient Requirements of Swine*. Washington, D. C.: National Academy Press, 1988, p. 93.

63. **Nikitin A, Egorov S, Daraselia N, Mazo I.** Pathway studio—the analysis and navigation of molecular networks. *Bioinformatics* 19: 2155–2157, 2003.
64. **Nkrumah JD, Keisler DH, Crews DH Jr, Basarab JA, Wang Z, Li C, Price MA, Okine EK, Moore SS.** Genetic and phenotypic relationships of serum leptin concentration with performance, efficiency of gain, and carcass merit of feedlot cattle. *J Anim Sci* 85: 2147–2155, 2007.
65. **Plas DR, Johnson R, Pingel JT, Matthews RJ, Dalton M, Roy G, Chan AC, Thomas ML.** Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. *Science* 272: 1173–1176, 1996.
66. **R Development Core Team.** *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing: Vienna, 2007.
67. **Renden JA.** Egg production efficiency in dwarf lines selected for high and low body weight as influenced by feed restriction. *Poult Sci* 66: 1085–1089, 1987.
68. **Richardson EC, Herd RM.** Biological basis for variation in residual feed intake in beef cattle. 2. Synthesis of results following divergent selection. *Aust J Exp Agric* 44: 431–440, 2004.
69. **Roberts AJ, Paisley SI, Geary TW, Grings EE, Waterman RC, MacNeil MD.** Effects of restricted feeding of beef heifers during the postweaning period on growth, efficiency, and ultrasound carcass characteristics. *J Anim Sci* 85: 2740–2745, 2007.
70. **Sadighi Akha AA, Miller RA.** Signal transduction in the aging immune system. *Curr Opin Immunol* 17: 486–491, 2005.
71. **Salmond RJ, Filby A, Qureshi I, Caserta S, Zamoyska R.** T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunol Rev* 228: 9–22, 2009.
72. **Schook L, Beattie C, Beever J, Donovan S, Jamison R, Zuckermann F, Niemi S, Rothschild M, Rutherford M, Smith D.** Swine in biomedical research: creating the building blocks of animal models. *Anim Biotechnol* 16: 183–190, 2005.
73. **Selman C, Kerrison ND, Cooray A, Piper MD, Lingard SJ, Barton RH, Schuster EF, Blanc E, Gems D, Nicholson JK, Thornton JM, Partridge L, Withers DJ.** Coordinated multitissue transcriptional and plasma metabonomic profiles following acute caloric restriction in mice. *Physiol Genomics* 27: 187–200, 2006.
74. **Sherman EL, Nkrumah JD, Murdoch BM, Moore SS.** Identification of polymorphisms influencing feed intake and efficiency in beef cattle. *Anim Genet* 39: 225–231, 2008.
75. **Shillabeer G, Vydelingum S, Hatch G, Russell JC, Lau DC.** Long-term regulation of leptin expression is correlated with adipocyte number in obese rats. *Clin Invest Med* 21: 54–62, 1998.
76. **Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R, Nair KS.** Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab* 283: E38–E43, 2002.
77. **Storey JD, Tibshirani R.** Statistical significance for genome-wide experiments. *Proc Natl Acad Sci USA* 100: 9440–9445, 2003.
78. **Suh Y.** Cell signaling in aging and apoptosis. *Mech Ageing Dev* 123: 881–890, 2002.
79. **Tuggle CK, Wang Y, Couture O.** Advances in swine transcriptomics. *Int J Biol Sci* 3: 132–152, 2007.
80. **Vainio H, Bianchini. F.** *Weight Control and Physical Activity. IARC Handbooks of Cancer Prevention 6.* Lyon, France: IARC Press, 2002, p. 1–23.

81. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes [Online]. *Genome Biol* 3: RESEARCH0034, 2002.
82. **Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE.** Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *J Clin Invest* 97: 2553–2561, 1996.
83. **Weindruch R, Kayo T, Lee CK, Prolla TA.** Microarray profiling of gene expression in aging and its alteration by caloric restriction in mice. *J Nutr* 131: 918S–923S, 2001.
84. **Wilson CL, Miller CJ.** Simpleaffy: a BioConductor package for Affymetrix Quality Control and data analysis. *Bioinformatics* 21: 3683–3685, 2005.
85. **Xie L, Jiang Y, Ouyang P, Chen J, Doan H, Herndon B, Sylvester JE, Zhang K, Molteni A, Reichle M, Zhang R, Haub MD, Baybutt RC, Wang W.** Effects of dietary calorie restriction or exercise on the PI3K and Ras signaling pathways in the skin of mice. *J Biol Chem* 282: 28025–28035, 2007.
86. **Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP.** Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 30: e15, 2002.
87. **Zha Y, Taguchi T, Nazneen A, Shimokawa I, Higami Y, Razzaque MS.** Genetic suppression of GH-IGF-1 activity, combined with lifelong caloric restriction, prevents age-related renal damage and prolongs the life span in rats. *Am J Nephrol* 28: 755–764, 2008.
88. **Zhang Q, Wang HY, Marzec M, Raghunath PN, Nagasawa T, Wasik MA.** STAT3- and DNA methyltransferase 1-mediated epigenetic silencing of SHP-1 tyrosine phosphatase tumor suppressor gene in malignant T lymphocytes. *Proc Natl Acad Sci USA* 102: 6948–6953, 2005.